AWARD NUMBER: W81XWH-14-1-0537

TITLE: Mobile, Multi-modal, Label-Free Imaging Probe Analysis of Choroidal Oximetry and Retinal Hypoxia

PRINCIPAL INVESTIGATOR: Dr. Stephen T.C. Wong

RECIPIENT: Houston Methodist Research Institute
Houston, TX 77030

REPORT DATE: October 2015

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2015	Annual	30 Sep 2014 - 29 Sep 2015

4. TITLE AND SUBTITLE
Mobile, Multimodal, Label-Free Imaging Probe Analysis of Choroidal Oximetry and Retinal Hypoxia

5a. CONTRACT NUMBER
W81XWH-14-1-0537

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
(1) Stephen T.C. Wong, PhD, PE & (2) Jared C. Gilliam, PhD

E-Mail: STWong@houstonmethodist.org & jgilliam2@houstonmethodist.org

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Methodist Hospital Research Institute
Gary Lingle
6565 Fannin St, STB 1-14
Houston, TX 77030-2703

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Coherent anti-stokes Raman spectroscopy (CARS) can be used to detect differences in the oxygen content in aqueous hemoglobin solutions. Our current setup is not ideal for accurately calibrating these measurements, but changes in the microscope setup and in the addition of gaseous oxygen to the physical sample are promising next steps.

15. SUBJECT TERMS
Primary blast injury, PBI, hypoxia, ischemia, oxygen, eye, retina, photoreceptor, neuron, TRPM7, neurodegeneration, neurotoxicity, coherent anti-Stokes Raman spectroscopy, CARS, mouse

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	10	19b. TELEPHONE NUMBER *(include area code)*

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1. INTRODUCTION:

Primary blast-induced injury (PBI) in combat veterans is correlated with a high degree of visual impairment that develops over time, even months to years after the initial injury was sustained. The cause of this visual function degradation is currently unknown, but it may be closely linked to the combination of chronic ischemia and resulting neurotoxicity that occur within the retina following many types of injury. Physical damage to the choroidal vasculature may reduce the amount of essential oxygen supplied to light-sensing photoreceptor neurons in the retina, leading to irreversible neurodegeneration and visual impairment. The purpose of this research is to investigate animal models of PBI in order to examine the potential link between trauma-induced hypoxia/ischemia in the choroid and altered cellular behavior in photoreceptor neurons that lead to photoreceptor cell death. By using coherent anti-Stoke Raman spectroscopy to measure choroidal blood oxygen levels following PBI, we aim to (1) test the potential link between trauma and vision loss, and (2) develop a mobile endoscope probe that has the capability of being used by clinicians to rapidly evaluate eye trauma following injury.

2. KEYWORDS:

Primary blast injury, PBI, hypoxia, ischemia, oxygen, eye, retina, photoreceptor, neuron, TRPM7, neurodegeneration, neurotoxicity, coherent anti-Stokes Raman spectroscopy, CARS, mouse

3. ACCOMPLISHMENTS:

Major Goals (Current Objectives):

1) Create PBI animal model	In Progress
2) Calibrate label-free probe for O_2 measurement	In Progress
3) Detect and map hypoxic regions in injured eyes	Not Started
4) Measure TRPM7 and cellular/apoptosis biomarkers in retinas	Not Started
5) Measure neuronal death and cell-specific biomarker in retinas	Not Started
6) Whole-cell electrophysiology for TRPM7 function	Not Started
7) Modulate TRPM7 activity in *ex vivo* retinas	Not Started

Major Task 1: Create PBI animal model (15% complete)
Subtask 1: Build pressurized-air blast device (40% complete)
Subtask 2: Build animal holding chamber (80% complete)
Subtask 3: Measure output pressure of device (25% complete)
Subtask 4: Apply (and optimize) controlled pressure to mice to induce primary blast injury (PBI) to eye. (0% complete)

Major activities carried out under this task have involved gaining IACUC approval of our animal protocol, constructing a pressure device that will be used in the future to inflict PBI using mice, and also constructing a holding chamber for mice to be used to restrain animals during the acute injury process.

Progress and Accomplishments:
After several rounds of review, our animal protocol has been approved by the HMRI IACUC committee. We have submitted the necessary documents for ACURO approval and are waiting for the process to be completed. After the ACURO is approved/granted, we will immediately begin work on finalizing the air-pressure gun and the animal holding chamber.

Subtask 1: In Progress
We are currently in the process of building a working version of this air-pressure gun and animal holding chamber that is suitable for work described in out IACUC animal protocol. After discussing the proposed blast procedure with the HMRI veterinary staff, we have made a few minor changes to the overall blast-gun design to accommodate an animal nose-cone for delivering real-time anesthesia in-procedure. We have made initial tests using a crude CO_2-air tank system in the laboratory to better understand how we should build the final air-pressure gun for use during animal experiments. As such, we have not completed the air-pressure gun, but we have identified the necessary parts required to make needed changes to the design. Parts are currently being ordered for this step.

Subtask 2: In Progress
We have designed and built some crude animal holding chambers out of various materials to test their durability during the experiments. We have identified a suitable material (polyvinyl chloride, PVC) that has the needed properties for the experiment as well as for sterilization properties for introducing the holder into the animal vivarium here at Houston Methodist Research Institute. We are still optimizing the build design and location of Velcro restraints and safety padding, which will need final approval from our veterinary staff before it can be used on animals.

Subtask 3: In Progress
We can measure air pressure from our crude setup, but it does not reflect the final air-gun setup at this point. We intend on building a custom-fitted machined barrel for our air-gun to increase output pressure range and increase stability/repeatability of the output prior to experimentation.

Subtask 4: Not started
We are currently waiting for our ACURO approval before we can begin animal research.

Discussion of Goals not Met:
Our biggest setback to date has been gaining IACUC approval of the animal protocol. The IACUC protocol has been approved and we have submitted the protocol for ACURO approval as well. We plan to complete the air-pressure gun and the animal holding chamber before the ACURO is completed. When animal research is able to begin, we will immediately begin work with the veterinary staff at HMRI to begin research as appropriate under our animal protocol. We expect both of these tasks can be completed, or mostly completed, before the ACURO process is complete and we expect this will allow us to move quickly into experiments in the future.

Major Task 2: Calibrate label-free probe for O_2 measurement
Subtask 1: Set up multi-modal label-free imaging probe system for use with mice. (50% complete)
Subtask 2: Calibrate probe for choroidal oximetry using CARS and SHG of hemoglobin (50% complete)

Major activities under this task are to set up our multi-modal label-free imaging system for use with mice and to calibrate our system to measure blood oxygen levels in the eye.

Progress and Accomplishments:

Subtask 1: In Progress
Modifications have been made to our current CARS microscope to accommodate mice during research. The biggest challenge currently is knowing how to apply a microscope objective to the mouse choroid for imaging while maintaining appropriate lubrication to the eye. This is an early step used during the initial calibration steps and is not intended for long-term experimental use during the study since experiments will be conducted using the label-free imaging probe. For the microscope setup: we have both water-immersion and air objectives suitable for CARS imaging, but each has its own set of strengths/weaknesses for experimentation regarding signal-to-noise ratios with ophthalmic ointments. To address this currently, we are testing ophthalmic ointments under the microscope to know, in advance of actual experiments, how different lubricants will affect image collection from the CARS microscope. We have successfully used such tests in the past in other studies and we expect this will be similar. In addition, we are currently running parallel tests using the label-free imaging probe to determine its signal sensitivity, absorbance, transmission, etc. towards ophthalmic ointments included in the proposed animal studies. Our probe is currently working in preliminary tests and we are eager to compare its signal to that of the microscope objectives we have used previously.

Subtask 2: In Progress
We have run initial tests on our CARS microscope using hemoglobin. Currently our results are inconsistent, because we have not optimized the protein resuspension method for use on the system. Currently we resuspend the protein in aqueous solution and place it on a microscope slide under a glass coverslip, but this process makes it difficult to infuse gaseous oxygen between the slide and coverslip to alter the oxy:deoxy hemoglobin ratio *in situ*. We are experimenting with a non-coverslip method of infusing oxygen and imaging the hemoglobin for calibration, but we have nothing concrete or reliably repeatable to report at this time.

Discussion of Goals not Met:
We previously reported problems with our coherent anti-Stokes Raman spectroscopy (CARS) laser system alignment that inhibited our progress on this area. The system has since been repaired and initial tests have begun using hemoglobin to calibrate the CARS laser system. We anticipate that the oxygen/hemoglobin calibration will be completed by the time the ACURO process is complete.

Major Task 3: Detect and map hypoxic regions in injured eyes (0% complete)
Subtask 1: Optimize animal intravital microscopy procedure
Subtask 2: Apply calibrated multi-modal probe to PBI test and control eyes and image choroidal vessels/capillaries using CARS intravital microscopy
Subtask 3: Measure oxy-hemoglobin levels in PBI test and control eyes using CARS and SHG
Subtask 4: Map regions of hypoxia in choroidal vasculature using CARS and SHG measurements

Current Status: Not Started

Discussion of Goals not Met:
This Major Task requires IACUC and ACURO approval to begin.

Major Task 4: Measure TRPM7 and cellular/apoptosis biomarkers in retinas (0% complete)
Subtask 1: Optimize detection of TRPM7 mRNA and protein in normal retinas using biochemical methods
Subtask 2: Optimize TRPM7 antibody labeling and detection by confocal microscopy and immunofluorescence
Subtask 3: Optimize detection of cell-specific biomarker mRNA and protein in normal retinas using biochemical and immunofluorescence methods
Subtask 4: Apply optimized TRPM7/biomarker methods to PBI test and control retinas

Current Status: Not Started

Discussion of Goals not Met:
This Major Task requires IACUC and ACURO approval to begin.

Major Task 5: Measure neuronal death and cell-specific biomarker in retinas (0% complete)
Subtask 1: Measure all biomarkers in PBI test and normal eyes

Current Status: Not Started

Discussion of Goals not Met:
This Major Task requires IACUC and ACURO approval to begin.

Major Task 6: Whole-cell electrophysiology for TRPM7 function (0% complete)
Subtask 1: Optimize retina slice preparation and elecrophysiological recording methods for TRPM7 channel activity.
Subtask 2: Optimize intracellular, patch-clamp imaging of fluorescent cationic indicators in *ex vivo* retina slices.

Current Status: Not Started

Discussion of Goals not Met:
This Major Task requires IACUC and ACURO approval to begin.

Major Task 7: Modulate TRPM7 activity in *ex vivo* retinas (0% complete)
Subtask 1: Modulate TRPM7 channel activity in PBI test and control retinas using published TRPM7 channel blockers.

Current Status: Not Started

Discussion of Goals not Met:
This Major Task requires IACUC and ACURO approval to begin.

Opportunities for Training: Nothing to Report
How were results disseminated to communities of interest? Nothing to Report

In order to accomplish our goals over the next reporting period:

Regarding the animal protocol: Our protocol has IACUC approval and has been submitted for ACURO review and approval. Animal experiments will not begin until this necessary approval has been granted. Developing the PBI animal model will begin after approval.

Regarding the blast device: we are in the process of purchasing the necessary components for this (discussed in conjunction with HMRI vet staff) and we will complete construction of this device. Many components are readily available by commercial suppliers and will be purchased directly. Some components will be altered or created uniquely for our build design. In this case, parts (such as the nozzle attachment of the blast device for adjusting output pressure) will be modified or build in our in-house machine shop.

Regarding the CARS probe calibration: We have purchased necessary chemicals (hemoglobin, oxygen, carbon dioxide) to calibrate our probe before ACURO approval is granted. We have already begun tests to calibrate our system, and we will continue to measure the dynamic CARS signal range obtained from aqueous hemoglobin solutions prepared with discreet percentages of O_2/CO_2. With these measurements, we will compute a system calibration curve. Our preliminary assumption is that this curve will serve as a reasonably model against which we can compare oxygen measurements obtained from PBI animals. We will test our assumption using animal data after animal-based experiments begin. Our immediate task is to find an appropriate method of measuring hemoglobin that does not use a microscope slide/coverslip system that will allow us to efficiently adjust O_2 concentration. Two methods will be tested (1) bubbling O_2 directly into the hemoglobin sample using surgical or microtubing, and (2) creating a batch of fully-oxygenated hemoglobin that will then be differentially added to calibration standards to create specific, known concentrations of oxygenated hemoglobin.

Regarding remaining items from Major Tasks 1 & 2: All other tasks are ready to begin as soon as ACURO approval is granted. We will begin these steps immediately upon approval.

Regarding all other Major Tasks: We will begin work on these immediately after ACURO approval is granted.

4. IMPACT: Nothing to Report

Impact on development of the principle discipline: Nothing to Report
Impact on other disciplines: Nothing to Report
Impact on technology transfer: Nothing to Report
Impact on society beyond science: Nothing to Report

5. CHANGES/PROBLEMS:

Changes in approach: Nothing to Report
Changes impacting expenditures: Nothing to Report
Significant changes in care of animals: Nothing to Report
Actual problems or delays:

We previously in Q2 of this year had laser alignment trouble with our CARS laser system. Currently the system is in working order, as of Q3 of this year, and we have begun using the system to collect calibration data. This setback was significant enough to delay some of our progress on Major Task 2. We anticipate no further delays in this regard.

6. PRODUCTS: Nothing to Report

Publications & presentations: Nothing to Report
Websites or other: Nothing to Report
Technologies: Nothing to Report
Inventions, patents, or licenses: Nothing to Report
Other products: Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS:

Individuals Working on Project: No Change

Name: Stephen T.C. Wong, PhD, PE
Project Role: Principle Investigator
Nearest person month worked: 1
Contribution to Project: Responsible for overall research direction

Name: Jared C. Gilliam, PhD
Project Role: Researcher/Postdoctoral Fellow
Nearest person month worked: 7
Contribution to Project: Responsible for developing all tools and procedures required for animal handling, microendoscopy system, measuring levels of TRPM7 in injured retinas, and measuring levels of TRPM7 activity within retinal neuron tissue.

Changes in in active support of senior personnel: Nothing to Report
Other organizations involved: Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS: None

9. APPENDICES: QuadChart

Vision Research Program - Hypothesis Development Award

MR130311 and Mobile Multi-Modal Label-free Imaging Probe Analysis of Choroidal Oximetry and Retinal Hypoxia

PI: Stephen T.C. Wong, Ph.D. P.E. **Org:** The Methodist Hospital Research Institute **Award Amount:** $250,000

Study/Product Aim(s)

- Calibrate prototype multi-modal label-free imaging probe for hemoglobin oximetry measurement
- Apply calibrated probe to animal PBI injury model
- Detect and map hypoxia regions in injured eyes
- Measure TRPM7 biomarker in mapped hypoxic eye regions
- Analyze TRPM7 activity with biochemistry and electrophysiology

Approach

Apply mobile multi-modal label-free imaging probe prototype to animal PBI models. Measure choroidal oxygen (proxy for ischemia) in injured eyes using probe.

Map hypoxic eye regions.

Measure altered TRPM7 expression (RNA, protein, biomarkers,etc) in the mapped hypoxic areas.

Determine role of Ca2+ and Mg2+ in TRPM7-induced photoreceptor death.

Insert a picture or graphic here, with a caption, that represents the proposed work

Accomplishment: Place a description of the latest scientific accomplishment here. Limit the comments to three lines or less to make them fit; be succinct. These comments are valuable since they show progress.

Timeline and Cost

Activities CY	13	14	15	16
1. Create PBI animal model				
2. Calibrate the label-free probe for measuring O2				
3. Map hypoxia in the eye using TRPM7 biomarker				
4. Measure and validate TRPM7 functions				
Estimated Budget ($K)	**$000**	**$45K**	**$130K**	**$75K**

Updated: (July 14, 2015)

Goals/Milestones

CY16 Goals – Animal and imaging systems set up and calibration

- ☐ Create PBI animal model for experimentation
- ☐ Complete the animal model by June 2016
- ☐ Set up mobile multi-modal label free imaging probe system
- ☐ Complete calibration and adjustment by February 2016
- ☐ Calibrate the label-free probe for choroidal oximetry

CY16 Goal – Measure and map hypoxia region in the eye

- ☐ Image, quantitate, and map hypoxia regions in the eye of PBI model
- ☐ Analyze TRPM7 gene expression in different hypoxia regions

CY16 Goal – Validate TRPM7 biomarker activities and functions

- ☐ Quantify TRPM7 channel electrodynamics using electrophysiology and Ca2+/Mg2+ imaging
- ☐ Validate the role of TRPM7 in choroidal oximetry and retinal hypoxia

Comments/Challenges/Issues/Concerns: NA

Budget Expenditure to Date

Projected Expenditure: $250K for two years

Actual Expenditure: NA